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01SEP98 E386923-3 D02890
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The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

REP05827GB

2. Patent application number
(The Patent Office will fill in this part)

9818915.2

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

KS Biomedix Ltd
42-46 High Street
Esher
Surrey
KT10 9QY
United Kingdom

Patents ADP number *(if you know it)*

07503808001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

ANTIBODIES

5. Name of your agent *(if you have one)*

GILL JENNINGS & EVERY

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)*

745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor
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c) any named applicant is a corporate body.
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YES

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Continuation sheets of this form	
Description	10
Claim(s)	2
Abstract	
Drawing(s)	1 X1

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Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	

11. For the Applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature: Gill Jennings & Every

Date: 28 August 1998

12. Name and daytime telephone number of person to contact in the United Kingdom

PERRY, Robert Edward
0171 377 1377

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ANTIBODIES

Field of the Invention

This invention relates to antibodies and their therapeutic use.

Background to the Invention

Antibodies have long been regarded as potentially powerful tools in the treatment of cancer and other diseases. However, although there have been some notable exceptions, this potential has not yet been realised.

This relative lack of success may be due, at least in part, to the use of monoclonal antibodies derived from rodents, which seldom have affinities higher than 10^{-9} M. Antibodies having this level of affinity are of limited therapeutic utility, as it has proved difficult to deliver enough antibody to the target to effect useful biological activity. Antibody binding to an antigen is reversible, and at the concentrations of antibody practical for *in vivo* use, dissociation will be favoured over association. In principle, it is possible to counter the dissociation of antigen by increasing the antibody concentration. However, this may lead to unacceptable clinical side-effects and would also increase the costs associated with the therapy.

Summary of the Invention

The present invention is based on the realisation that antibodies, or fragments thereof, can be produced which are "acid-resistant" and that this property is associated with high affinity binding of an antibody for its antigen.

According to the present invention, a high-affinity antibody has affinity characterised by:

(i) incubating first and second samples of the antibody in antigen-coated microtitre plate wells at a concentration chosen to be within the linear response part of a standard curve at pH 7.2 for 1 hour at 37°C;

(ii) removing unbound antibody from both samples;

(iii) incubating the first sample with PBS at pH 7.2 for 1 hour at 37°C, and reducing the pH of the second sample to pH 2 and incubating for 1 hour at 37°C;

(iv) removing unbound antibody from both samples;

(v) incubating both samples with anti-antibody alkaline-phosphatase conjugate for 1 hour at 37°C;

(vi) removing unbound conjugate from both samples; and

(vii) adding PNPP substrate to the samples, measuring absorbance of the samples at 405nm, and determining the amount of antibody bound to antigen, wherein the amount bound in the second sample is >50% of that of the first sample.

Antibodies or antibody fragments with the "acid-resistant" properties are expected to favour association rather than dissociation and they therefore have longer localisation times at target sites which results in a higher concentration of antibodies localised at the target sites.

In particular this invention relates to the production of a high affinity single-chain Fv antibody fragment. This ScFv has particular advantages in that it allows better targeting to a site *in vivo*.

Description of the Drawing

Figure 1 illustrates the results achieved for acid-resistance of sheep and mouse monoclonal antibodies and single-chain Fvs with affinity to carcinoembryonic antigen at various pH values.

Description of the Invention

The acid-resistant monoclonal antibodies according to the present invention may be obtained using various techniques. For example, classical hybridoma technology can be applied, comprising the fusion of B-lymphocytes from immunised animals secreting high-affinity antibodies with an appropriate fusion partner. An alternative method is to purify the mRNA from selected lymphocytes and use the technique of PCR to amplify the antibody genes required. Phage display technology may also be used to obtain the antibody genes from naive or immunised libraries after appropriate selection procedures.

The antibody gene can be co-expressed with or otherwise chemically linked to toxins, radioisotopes or enzymes or any other desirable molecules to provide a fusion protein with strong binding characteristics.

The antibody may be a whole antibody, comprising heavy and light chains, and constant and variable regions. Alternatively, the antibody is an antibody fragment, e.g. $F(ab')_2$, Fab, Fv or single-chain Fv fragments, provided that at least part of the variable region is present which confers the property of "acid resistance".

In a preferred embodiment of the invention, the antibody is a single-chain Fv fragment. The single-chain Fv fragment comprises both heavy chain and light chain variable regions linked by a suitable peptide.

The antibodies of the present invention may be defined by their acid-resistant properties, which can be characterised by an acid washed ELISA, as described above. Typically the A_{405} value obtained by ELISA will represent antibody binding of >50% for a sample at pH 2, compared to the value for the sample at pH 7.2. Preferably, the A_{405} value of the sample at pH 2 will represent antibody binding of >60% of that obtained at pH 7.2.

The animal that is subjected to immunisation is not a rodent, but is chosen to give higher affinity antibodies. Any large mammal may be used and suitable animals include rabbits, goats, cows and sheep.

An antibody of the invention may be used in therapy and may be formulated into any suitable composition with a physiologically-acceptable excipient, diluent or carrier.

The following Examples illustrate the invention.

Example 1.

Sheep were immunised with carcinoembryonic antigen (CEA) in complete Freund's adjuvant, then boosted three times with antigen in incomplete Freund's adjuvant. Animals were sacrificed after the final boost and lymph nodes removed.

The lymph node cells were then washed and fused with sheep heteromyeloma fusion partner SFP3.2. Fused cells were plated out at a total density of approximately 10^6 per ml in medium containing HAT (Life Technologies). These samples were then screened for hybridomas secreting high-affinity antibodies to the specified antigen using both a normal ELISA and an acid washed ELISA.

Standard ELISA screening assays were carried out as follows:

Maxisorb assay plates (NUNC) were coated with CEA (0.4 ug/ml in phosphate buffered saline at pH 7.2), 100µl per well and left overnight at 4°C. The plates were then washed three times using phosphate buffered saline at pH 7.2 with 0.01% Tween 20 detergent. Any remaining reactive sites on the plates were blocked by the addition of 200µl per well of 0.2% fat-free milk protein in PBS at pH 7.2 at 37°C for ½ hour. The plates were then washed in PBS as described above and 45µl of the antibody samples were added to the wells of the plates. The samples were incubated for one hour at 37°C and then washed as described previously. Bound antibody was detected using alkaline phosphatase-conjugated donkey anti-sheep antibody (Sigma A5187 diluted 1/5000 in PBS at pH 7.2 with 1% BSA). The plates were then washed and 100µl per well of PNPP (Sigma N2770) solution was added. Absorbance was measured using a spectrophotometer at 405nm with phosphate buffered saline as a control.

Acid wash ELISA screening assays were carried out as follows:

Coating and binding of antibody samples was as described for the standard ELISA above. However, after incubation with the antibody samples, the plates were washed and 200µl per well of HCl (10mM Stock solution) at pH 2 was added for one hour at 37°C. After three washes the antibody remaining bound to antigen was detected using alkaline-phosphatase-conjugated donkey anti-sheep antibody and PNPP as described above. In order to ensure that a

proper comparison was being made between antibodies at different concentrations, each sample was chosen to give an A_{405} value of approximately 1.0 in the normal ELISA (i.e. in the linear response part of the ELISA curve).

Three hybridomas (1D2, 6G11 and 6H9) secreted antibodies which gave a greater than 50% retention of binding in the acid washed ELISA, in comparison to the binding in the non-acid washed ELISA.

Example 2

A single-chain Fv fragment was produced from the hybridoma 6H9 above, as follows:

mRNA was purified from the cultured hybridoma cells using oligo-dT cellulose. Single-stranded DNA complementary to the mRNA (cDNA) was synthesized by reverse transcription. Universal primers, designed from the constant regions of sheep heavy and light chain antibody genes, were used in separate reverse transcription reactions to synthesise the cDNA for the antibody variable regions.

The cDNA was then amplified by the polymerase chain reaction to make double-stranded DNA using primers designed from the heavy and light chain variable framework sequences. Separate polymerase chain reactions were used to amplify the heavy and light chain regions. The products were then analysed by agarose gel electrophoresis and the DNA bands equivalent to light and heavy chain genes were cut from the gel and purified.

Equimolar amounts of variable heavy and light chain DNA were mixed together with an oligonucleotide linker DNA. The linker DNA coded for the amino acid sequence $(Gly_4Ser)_3$ with additional nucleotides complementary to the 3′ end of the heavy chain variable region and the 5′ end of the light chain variable region. The three DNA molecules were denatured, annealed and extended in the first stage (without primers) of a two-stage PCR reaction so that the fragments were joined, thereby assembling the single-chain Fv.

The single-chain Fv DNA was amplified in the second stage of the PCR using a pair of primers derived from the heavy and light chain variable region termini with the addition of the restriction enzyme recognition sites for AlW44i and NotI. The single-chain Fv gene product was analysed by agarose gel electrophoresis and purified. The single-chain Fv was then digested with the restriction enzymes AlW44i and NotI and cloned into an expression vector. The vector was then used to transform *E. coli* HB 2151, and protein expression was allowed to occur. The vector was designed so as to include a hexa-histidine tag at the COOH terminus of the SFv. The single-chain Fv was purified using nickel-chelate affinity chromatography and analysed by SDS-PAGE. The amino acid sequence for the heavy chain variable region and the light chain variable region is disclosed in SEQ ID Nos. 2 and 4, respectively. An acid wash ELISA was also carried out to determine the acid-resistant properties of the single-chain Fv.

Acid wash ELISA was carried out as follows:

Carcinoembryonic antigen (CEA) coated microtitre plates were prepared as described previously. Single-chain Fv samples (6H9) were diluted to a range of concentrations between 1ng/ml and 100ng/ml in PBS at pH 7.2 containing 1% BSA. 100µl samples were added to the microtitre plate wells and incubated for 1 hour at 37°C. The plates were then washed, 200µl per well of citrate added, and the plates incubated for 1 hour at 37°C. In this case, the acid preparations were made using a stock solution of 100mM citrate diluted to pHs 4.0, 3.5, 3.0, 2.5 and 2.0 in the reaction mixture. PBS at pH 7.2 was used as a reference control. The plates were then washed and 100µl per well of mouse anti-tetra-histidine antibody (Qiagen) (100ng/ml diluted in PBS at pH 7.2 with 1% BSA) added and incubated for 1 hour at 37°C. After plate washing the samples were incubated for 1 hour at 37°C with 100µl per well of goat anti-mouse alkaline phosphatase conjugate (Sigma A3688 diluted 1/1000 in PBS with 1% BSA at pH 7.2). The plates

were then washed, treated with PNPP as described previously and the absorbance measured using a spectrophotometer at 405nm.

As a control for acid resistance, sFv samples were incubated with PBS at pH 7.2 to generate an ELISA response curve for the SFv samples. In the linear region, a concentration of 10-20ng/ml of the SFv sample gave an absorbance (A_{405}) of 1.0-1.5 and was therefore used to determine the amount of antibody bound in the acid washed samples as a percentage of the amount bound in the reference sample.

The acid-resistant properties of the 6H9 whole antibody and the 6H9 single-chain Fv were compared with that for the mouse-derived anti-carcinoembryonic antigen whole antibody, A5B7 and the single-chain Fv MFE. The results are shown in Figure 1, with the antigen-binding of the mouse-derived antibodies being substantially reduced at pH 3.5 and less than 5% at pH 2.5. In contrast, the 6H9 antibodies retain >70% antigen at pH 3.5, >60% at pH 2.5 and >50% at pH 2.0.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: KS Biomedix Limited
(B) STREET: 42-46 High Street
(C) CITY: Esher
(D) STATE: Surrey
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): KT10 9QY

(ii) TITLE OF INVENTION: Antibodies

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 363 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..363

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CAG GTG CAG CTG CAG GAG TCG GGA CCC AGC CTG GTG AAG CCC TCA CAG          48
Gln Val Gln Leu Gln Glu Ser Gly Pro Ser Leu Val Lys Pro Ser Gln
 1               5                  10                  15

ACC CTC TCC CTC ACC TGC ACG GTC TCT GGA TTC TCA TTA ACC AAG TAT          96
Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Lys Tyr
            20                  25                  30

GGT GTT AGT TGG GTC CGC CAG GCT CCA GGA AAG GCG CTT GAG TGG CTA         144
Gly Val Ser Trp Val Arg Gln Ala Pro Gly Lys Ala Leu Glu Trp Leu
        35                  40                  45

GGT GGT GTG TCC AGT GGT GCA CTA ACA GCC TAT AAC ACA GCC CTA CAG         192
Gly Gly Val Ser Ser Gly Ala Leu Thr Ala Tyr Asn Thr Ala Leu Gln
    50                  55                  60

TCC CGA CTC AGC GTC ACC AGG GAC ACC TCC AAG AGC CAA TTC TCC CTG         240
Ser Arg Leu Ser Val Thr Arg Asp Thr Ser Lys Ser Gln Phe Ser Leu
65                  70                  75                  80

TCA CTG AGC AGC GTG ACT ACT GAG GAC ACG GCC ATT TAC TAC TGT GCG         288
Ser Leu Ser Ser Val Thr Thr Glu Asp Thr Ala Ile Tyr Tyr Cys Ala
                85                  90                  95
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AAA TCT GTC AAT GGT GAC AGT GTT CCT TAT GGT TTG GAC TAC TGG AGC        336
Lys Ser Val Asn Gly Asp Ser Val Pro Tyr Gly Leu Asp Tyr Trp Ser
            100                 105                 110

CCA GGA CTC CTA CTC ACC GTC TCC TCA                                   363
Pro Gly Leu Leu Leu Thr Val Ser Ser
        115                 120
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 121 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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 1               5                  10                  15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Thr Lys Tyr
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Gly Val Ser Trp Val Arg Gln Ala Pro Gly Lys Ala Leu Glu Trp Leu
        35                  40                  45

Gly Gly Val Ser Ser Gly Ala Leu Thr Ala Tyr Asn Thr Ala Leu Gln
    50                  55                  60

Ser Arg Leu Ser Val Thr Arg Asp Thr Ser Lys Ser Gln Phe Ser Leu
65                  70                  75                  80

Ser Leu Ser Ser Val Thr Thr Glu Asp Thr Ala Ile Tyr Tyr Cys Ala
                85                  90                  95

Lys Ser Val Asn Gly Asp Ser Val Pro Tyr Gly Leu Asp Tyr Trp Ser
            100                 105                 110

Pro Gly Leu Leu Leu Thr Val Ser Ser
        115                 120
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..333

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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CAG GAT GTG CTG ACT CAG CCG TCC TCC GTG TCT GGG TCC CTG GGC CAG        48
Gln Asp Val Leu Thr Gln Pro Ser Ser Val Ser Gly Ser Leu Gly Gln
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AGG GTC TCC ATC ACC TGC TCT GGA AGC AGC AGC AAC ATT GGA GGT AAT      96
Arg Val Ser Ile Thr Cys Ser Gly Ser Ser Ser Asn Ile Gly Gly Asn
        140                 145                 150

GCT TAT GTG GGC TGG TAC CAA CAG GTC CCA GGA TCA GCC CCC AGA CTC     144
Ala Tyr Val Gly Trp Tyr Gln Gln Val Pro Gly Ser Ala Pro Arg Leu
    155                 160                 165

CTC ATC AGT GCT ACA ACC GAT CGA GCC TCG GGG ATC CCC GAC CGA TTC     192
Leu Ile Ser Ala Thr Thr Asp Arg Ala Ser Gly Ile Pro Asp Arg Phe
170                 175                 180                 185

TCC GGC TCC AGG TCT GGG AAC ACA GCC ACC CTG ACC ATC AGC TCG CTC     240
Ser Gly Ser Arg Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Ser Leu
                190                 195                 200

CAG GCT GAG GAC GAG GCC GAT TAT TAC TGT GCA TCG TAT CAA AGT ACT     288
Gln Ala Glu Asp Glu Ala Asp Tyr Tyr Cys Ala Ser Tyr Gln Ser Thr
            205                 210                 215

TAC AGT GGT GTT TTC GGC AGC GGG ACC AGG CTG ACC GTC CTG GGT         333
Tyr Ser Gly Val Phe Gly Ser Gly Thr Arg Leu Thr Val Leu Gly
        220                 225                 230
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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 1               5                  10                  15

Arg Val Ser Ile Thr Cys Ser Gly Ser Ser Ser Asn Ile Gly Gly Asn
            20                  25                  30

Ala Tyr Val Gly Trp Tyr Gln Gln Val Pro Gly Ser Ala Pro Arg Leu
        35                  40                  45

Leu Ile Ser Ala Thr Thr Asp Arg Ala Ser Gly Ile Pro Asp Arg Phe
    50                  55                  60

Ser Gly Ser Arg Ser Gly Asn Thr Ala Thr Leu Thr Ile Ser Ser Leu
65                  70                  75                  80

Gln Ala Glu Asp Glu Ala Asp Tyr Tyr Cys Ala Ser Tyr Gln Ser Thr
                85                  90                  95

Tyr Ser Gly Val Phe Gly Ser Gly Thr Arg Leu Thr Val Leu Gly
            100                 105                 110
```

CLAIMS

1. A high-affinity monoclonal antibody, wherein the affinity is characterised by:

(i) incubating first and second samples of the antibody in antigen coated microtitre plate wells at a concentration chosen to be within the linear part of a standard curve at pH 7.2 for 1 hour at 37°C;

(ii) removing unbound antibody from both samples;

(iii) incubating the first sample with PBS at pH 7.2 for 1 hour at 37°C, and reducing the pH of the second sample to pH 2 and incubating for 1 hour at 37°C;

(iv) removing unbound antibody from both samples;

(v) incubating both samples with anti-antibody alkaline phosphatase-conjugate for 1 hour at 37°C;

(vi) removing unbound conjugate from both samples; and

(vii) adding PNPP substrate to the samples, measuring the absorbance of the samples at 405nm, and determining the amount of antibody bound to antigen, wherein the amount bound in the second sample is >50% of that of the first sample.

2. An antibody according to claim 1, wherein the amount of antibody bound in second sample is >60% of that bound in the first sample.

3. An antibody according to claim 1 or claim 2, which is non-rodent.

4. An antibody according to any preceding claim, which has affinity for a tumour-associated antigen.

5. An antibody according to claim 4, wherein the antigen is carcinoembryonic antigen.

6. An antibody according to any preceding claim, which is a single-chain Fv.

7. An antibody according to claim 6, having a heavy chain variable region comprising the amino acid sequence defined in SEQ ID No. 2 and a light chain variable region comprising the amino acid sequence defined in SEQ ID No. 4, or a variant thereof.

8. A polynucleotide molecule encoding an antibody according to claim 7, wherein the polynucleotide comprises a nucleotide sequence defined in SEQ ID Nos. 1 and 3, or a variant thereof.

9. A cloning vehicle comprising the polynucleotide molecule according to claim 8.

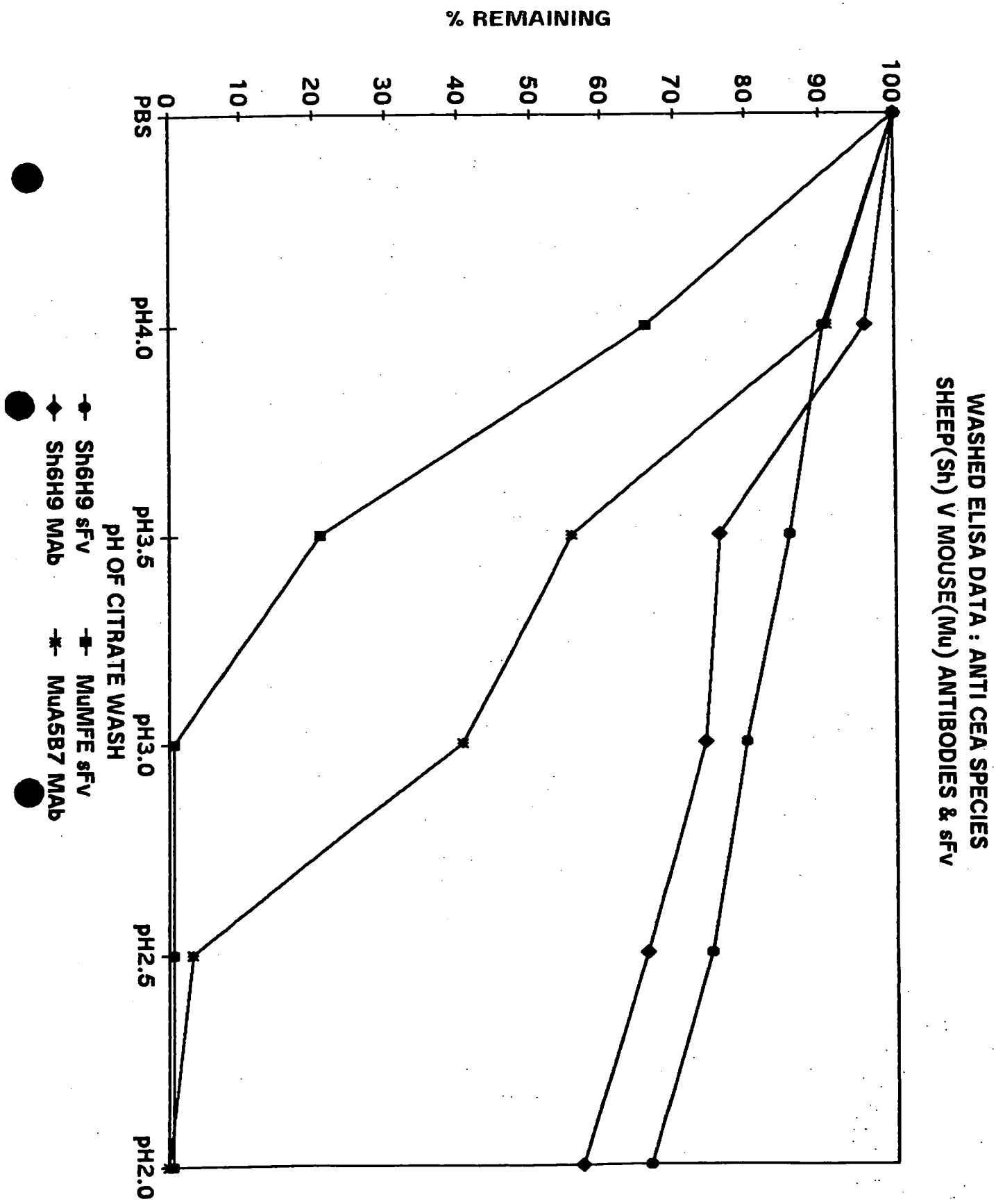
WASHED ELISA DATA : ANTI CEA SPECIES
SHEEP(Sh) V MOUSE(Mu) ANTIBODIES & sFv
% REMAINING
0
10
20
30
40
50
60
70
80
90
100
PBS
pH4.0
pH3.5
pH3.0
pH2.5
pH2.0
pH OF CITRATE WASH
Sh6H9 sFv
Sh6H9 MAb
MuMFE sFv
MuA5B7 MAb

FIG. 1

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AGENT : Gill Jennings & Every

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